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**Title:** Candidalysin drives epithelial signaling, neutrophil recruitment, and immunopathology at the vaginal mucosa.

**Running title:** Candidalysin and vaginitis

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## ABSTRACT

Unlike other forms of candidiasis, vulvovaginal candidiasis, caused primarily by the fungal pathogen *Candida albicans*, is a disease of immunocompetent and otherwise healthy women. Despite its prevalence, the fungal factors responsible for initiating symptomatic infection remain poorly understood. One of the hallmarks of vaginal candidiasis is the robust recruitment of neutrophils to the site of infection, which seemingly do not clear the fungus, but rather exacerbate disease symptomatology. Candidalysin, a newly discovered peptide toxin secreted by *C. albicans* hyphae during invasion, drives epithelial damage, immune activation and phagocyte attraction. Therefore, we hypothesized that Candidalysin is crucial for vulvovaginal candidiasis immunopathology.

Anti-*Candida* immune responses are clearly anatomical site specific, as effective gastrointestinal, oral, and vaginal immunity is uniquely compartmentalized. Thus, we aimed to identify the immunopathologic role of Candidalysin and downstream signaling events at the vaginal mucosa. Microarray analysis of *C. albicans*-infected human vaginal epithelium *in vitro* revealed signaling pathways involved in epithelial damage responses, barrier repair, and leukocyte activation. Moreover, treatment of A431 vaginal epithelial cells with Candidalysin induced dose-dependent pro-inflammatory cytokine responses (including IL-1 $\alpha$ , IL-1 $\beta$  and IL-8), damage, and activation of c-Fos and mitogen activated protein kinase (MAPK) signaling, consistent with fungal challenge. Mice intravaginally challenged with *C. albicans* strains deficient in Candidalysin exhibited no differences in colonization or hyphal burdens as compared to isogenic

controls. However, significant decreases in neutrophil recruitment, damage, and pro-inflammatory cytokine expression were observed with these strains. Our findings demonstrate that Candidalysin is a key hypha-associated virulence determinant governing the immunopathogenesis of *C. albicans* vaginitis.

## INTRODUCTION

Vulvovaginal candidiasis (VVC), caused primarily by the polymorphic fungal pathogen *Candida albicans*, remains a serious worldwide health concern leading to significant quality of life issues for immunocompetent women (1). Symptomatic VVC is manifested by itching, burning, and pain sensation at the vaginal and vulvar tissue, often accompanied by odorless vaginal discharge (2). Globally, VVC is estimated to be the most prevalent human fungal infection, with over 75% of women experiencing at least one episode in their lifetime and 5-8% suffering from recurrent infection (3). In recent years, VVC has been described as an immunopathology, in which the host neutrophil response actually exacerbates disease symptoms, yet fails to adequately control the fungus (4, 5). While much effort has been placed on defining host immunological mechanisms contributing to VVC protection, the fungal virulence factors that dictate conversion from asymptomatic colonization to fulminant infection remain poorly understood.

Using model systems, several laboratories have collectively begun to unravel this complex host-pathogen interaction. Studies revealed that vaginal epithelial cell damage and immune activation is dependent on *C. albicans* hypha formation, with epithelial immunity being mediated via the MAPK transcription factor c-Fos and regulated by the

MAPK phosphatase MKP1 (6). This MAPK/c-Fos/MKP1 pathway is able to discriminate between *C. albicans* yeast and hyphal cells and is retained between both vaginal and oral epithelia (7, 8). The activation of this signaling pathway is also coupled with lactate dehydrogenase (LDH) release, a marker of epithelial damage (6). Therefore, the ability of *C. albicans* to form hyphae and breach the epithelial barrier governs innate cytokine release and pro-inflammatory responses.

Using a clinically relevant murine model of VVC, it was also determined that genetic blockade of hypha formation (e.g. *efg1Δ/Δ*, *efg1Δ/Δ/cph1Δ/Δ*, *NRG1* overexpression) failed to induce hallmark immunopathology at the vaginal mucosa, including polymorphonuclear leukocyte (PMN/neutrophil) recruitment, S100A8 release, IL-1 $\beta$  production, and LDH release confirming a crucial role for hyphae in this disease (9). Moreover, depletion of neutrophils *in vivo* failed to reduce LDH release from mice infected with hypha-competent strains of *C. albicans* (9, 10). Although, depletion of PMNs did significantly reduce several hallmark cytokines and chemokines during infection. These data suggest that *C. albicans* directly damages the vaginal mucosa which elicits migratory neutrophils as secondary effectors that amplify symptomatic vaginal inflammation. However, the fungal factors and mechanisms that contribute to neutrophil recruitment and induction of immunopathology remain elusive. In general, these experiments recapitulated findings previously observed using human vaginal epithelial cell lines (6). Using a combination of RNA-Seq and targeted knockout studies, it was demonstrated that the NLRP3 inflammasome, a host signaling complex activated in response to cellular damage, is crucial for IL-1 $\beta$  release and PMN recruitment to the vaginal mucosa during *C. albicans* infection, consistent with candidal infection at other

anatomical sites (11). However, an important question remained: is hypha formation *per se* sufficient for damage-induced inflammation or are distinct fungal factors associated with the morphogenetic transition involved in these processes?

Until recently, individual gene deletions in *C. albicans* have demonstrated only modest phenotypic alterations with respect to fungal colonization or cytokine signaling at the vaginal mucosa; the greatest reported effects being with deletion of the secreted aspartyl proteinase genes, *SAP2* and *SAP5* (11-14). However, recent work has revealed that the *C. albicans ECE1* (extent of cell elongation) gene product is crucial for cellular damage, innate cytokine production, and neutrophil recruitment during murine oropharyngeal candidiasis (OPC). *ECE1*, a highly expressed, hypha-associated gene encodes a protein (Ece1p) that is processed into eight distinct peptides by the fungal protease Kex2p (15, 16). Genetic, biochemical and functional assays determined that amino acids 62-92 of Ece1p encode a fungal toxin termed Candidalysin, which possesses both lytic and immunostimulatory activity (including MAPK signaling) on oral epithelial cells (16). Importantly, an *ece1* $\Delta/\Delta$  null mutant retains the capacity to form hyphae yet is unable to induce an inflammatory response. Moreover, *ECE1* expression is largely dependent on the Efg1 transcription factor, likely explaining why *efg1* $\Delta/\Delta$  and *efg1* $\Delta/\Delta$ /*cph1* $\Delta/\Delta$  mutants fail to fully activate MAPK signaling and immunopathology in oral or vaginal epithelium (6, 8, 9, 17).

Given these similarities, we hypothesized that Candidalysin may comparably activate vaginal epithelial cells and govern VVC immunopathology *in vivo*. This study demonstrates that a single fungal factor, Candidalysin, is responsible for inducing vaginal cellular damage and pro-inflammatory responses during *C. albicans* infection *in*

*vitro* and *in vivo*. As such, the identification of a secreted toxin as the factor responsible for driving symptomatic vaginal inflammation may offer novel treatment modalities for arresting symptomatic disease.

## RESULTS

### Differential gene expression and pathway induction in reconstituted human

**vaginal epithelium following *C. albicans* challenge.** The reconstituted human vaginal epithelium (RVE) model is an excellent *in vitro* surrogate to study epithelial-specific responses of vaginal candidiasis, as the tissue layer is sufficiently differentiated and supports robust hyphal invasion and infected RVE tissue largely resemble the *in vivo* situation (18, 19). In order to elucidate global host transcriptomic changes in vaginal epithelium in response to challenge with *C. albicans* as compared to PBS sham control, total epithelial RNA was selectively isolated from RVE at 6 and 24 h post-challenge and subjected to microarray analysis. As with oral epithelium, the intermediate (6 h) time point is associated with initial fungal adherence and detection, while the late (24 h) time point is associated with fungal invasion and cellular damage (8, 20). Approximately 800 and nearly 4,000 genes were differentially expressed ( $P < 0.001$ ) at least 2-fold at 6 and 24 h, respectively in response to *C. albicans* (Fig. 1). Comparatively, few genes were regulated in response to PBS-sham treatment at the same time points (Fig. S1). At the intermediate stage of infection (6 h post-infection), the majority of differentially expressed genes are up-regulated (Fig 1A) with none showing strong down-regulation. However, by late stages of infection (24 h), there is an increase in the proportion of genes showing down-regulated expression (Fig 1B). Over half of the genes showing up-

regulation at 6 h were similarly up-regulated at 24 h (Fig 1C). Surprisingly, relatively few genes were strongly (>4 fold) down-regulated in response to fungal challenge at either time point. Commensurate with these associations, both time points include genes associated with MAPK (phosphatases, accessory proteins and transcription factors), NF- $\kappa$ B and PI3K signaling. The early time point includes the expression of many genes encoding early/immediate transcription factors (such as c-Fos) and signaling modulators (such as the DUSP phosphatases) that are associated with the immediate signaling response to the presence of *C. albicans*. However, the late time point contains a far more diverse set of genes, such as downstream effector genes, including cytokines. This pool represents the cumulative responses driven by the continued presence of *C. albicans* hyphae along with subsequent released host factors. At both time points, the up-regulated expression of a variety of transcription factor genes associated with other signaling pathways was observed, as well as genes coding for cytokines and effector molecules.

Gene ontology, pathway, and network mapping using the web-based analysis package DAVID revealed profiles from *C. albicans* infected cells as consistent with MAPK, NF- $\kappa$ B PI3K, ErbB receptor, and TNF signaling pathways (Fig. 2). Pathways involving extracellular matrix remodeling, including proteoglycans in cancer, focal adhesion, adherens junctions, and tight junctions were also significantly enriched during *C. albicans* infection. Pathways involved in responses to infection by other microbes, including Epstein-Barr virus, *Shigella*, Hepatitis B, Influenza A, Herpes virus, *Salmonella*, and trypanosome infection were also predicted to be activated, suggesting conservation of epithelial responses against a broad array of pathogens. Pathways



169 predicted to be activated were generally conserved at 6 h and 24 h time points. A list of  
170 individually expressed genes may be found in Table S1.

171 Genes involved in innate inflammatory signaling were strongly induced by  
172 *C. albicans*, including those encoding cytokines *IL-8* (100-fold), *IL-1A* (18-fold), *IL-1B*  
173 (3.8-fold), *CXCL1* (19-fold), *CXCL2* (26-fold), *GM-CSF* (10-fold), and prostaglandin  
174 synthase *PTGS2* (7.3-fold), many of which play critical roles in recruiting inflammatory  
175 cells (particularly neutrophils) to the site of infection. Similar to previous findings, there  
176 was clear induction of genes associated with MAPK activity: *MAP3K2* (6.8-fold),  
177 *MAP2K3* (4-fold), *MAP3K9* (4-fold), *MAP4K4* (2.7-fold). Additionally, *C. albicans*  
178 infection led to epithelial induction of *c-FOS* (32-fold) and *c-JUN* (17.7-fold), which  
179 encode members of two families that form the heterodimeric transcription factor AP1, a  
180 major effector of MAPK activation. The dual specificity phosphatase 1 (*DUSP1*) gene,  
181 encoding a regulator of MAPK signaling, was also elevated (6.7-fold) in response to *C.*  
182 *albicans*.

183 A number of other genes were induced that are involved in tissue repair, wound  
184 healing, or dampening of active inflammation, including the genes coding for IL-24 (2.3-  
185 fold) and IL-1RN (4-fold) were increased during *C. albicans* infection (21, 22).  
186 Interestingly, a number of other related genes were also induced, including genes  
187 coding for HBEGF (heparin binding EGF-like growth factor, 39.5-fold) and EREG  
188 (epiregulin, 6-fold) that are members of the epidermal growth factors (EGFs). They exert  
189 their function by binding to their cognate receptors EGFR or v-erb-b2 oncogene  
190 homolog (ERBB) to induce cellular proliferation and healing of skin and epidermal  
191 tissues (23, 24).

**Candidalysin damages and activates vaginal epithelial cells.** As we observed an up-regulated expression of genes encoding several pro-inflammatory cytokines (e.g. *IL-1A*, *IL-1B*, *IL-8*, *GM-CSF*) and chemokines during RVE challenge with *C. albicans* at time points when hyphae invaded the vaginal tissue, we sought to determine whether the hyphae associated peptide toxin Candidalysin similarly elicited these effector and damage responses. Indeed, there was a dose-dependent release of lactate dehydrogenase (LDH) when Candidalysin was applied to A431 cells (Fig. 3A). Significant levels of cellular damage were observed with doses above 15  $\mu$ M as compared to treatment with the vehicle control.

Vaginal epithelial cells respond to *C. albicans* hyphae by activating the p38-MAPK and ERK1/2-MAPK signalling pathways, resulting in the regulated secretion of proinflammatory cytokines (6). However, the fungal factors governing epithelial activation are unknown. To assess whether Candidalysin is capable of activating p38-MAPK and ERK1/2-MAPK signalling pathways, epithelial cells were exposed to Candidalysin *in vitro*, and c-Fos production/MKP1 phosphorylation was assessed by Western blotting (Fig. 3B). The c-Fos/p-MKP1 response was induced strongly by 70 and 15  $\mu$ M Candidalysin, whereas the vehicle was unable to activate signalling. Concomitant with damage, treatment with Candidalysin caused a dose-dependent increase in the release of IL-1 $\alpha$ , IL-1 $\beta$ , G-CSF, GM-CSF and IL-8 in spent culture supernatants (Fig. 3C-H). The lone exception was IL-6, which was only significantly elevated at the highest Candidalysin concentration (70  $\mu$ M). With the exception of IL-6, all cytokines assayed were significantly induced at Candidalysin doses above 3  $\mu$ M; however, this dose was

insufficient to cause significant damage (Fig. 3A), suggesting that Candidalysin exhibits dual functionality, serving both immunostimulatory and lytic roles against vaginal epithelial cells, similar to that observed in oral epithelia (16).

**Candidalysin is required for vaginitis immunopathology.** We next questioned whether Ece1 and/or Candidalysin contribute to immunopathology in an established estrogen-dependent mouse model of vulvovaginal candidiasis (VVC). Therefore, we utilized strains of *C. albicans* that had been deleted for both copies of *ECE1* (*ece1Δ/Δ*) and restored with one full-length allele (*ece1Δ/Δ+ECE1*) or one mutant allele lacking the Candidalysin-encoding region of *ECE1* (*ece1Δ/Δ+ECE1<sub>Δ184-279</sub>*), along with the appropriate parental isogenic control (BWP17+Clp30: from here referred to as “WT”). Somewhat surprisingly, recovered fungal burdens from the vaginal lavage fluid were not significantly different between strains at either d 3 (Fig. 4A) or d 7 (Fig. 4B) post-inoculation. However, there was a significant reduction in the number of neutrophils recruited into the vaginal lumen during challenge with either *ece1Δ/Δ* or *ece1Δ/Δ+ECE1<sub>Δ184-279</sub>* strains, which was restored to WT levels during infection with the *ece1Δ/Δ+ECE1* re-integrand strain (Fig. 4C,D,G, yellow arrows). Consistent with this phenotype, levels of the damage biomarker LDH were significantly reduced with these same mutants as compared to infection with WT or *ece1Δ/Δ+ECE1* re-integrand (Fig. 4E,F). Given our previous data using hypha deficient strains, a morphogenesis defect may account for this phenotype (9). However, *ece1Δ/Δ* and *ece1Δ/Δ+ECE1<sub>Δ184-279</sub>* strains formed hyphae normally at the vaginal mucosa, as did WT and *ece1Δ/Δ+ECE1* strains (Fig. 4G, green arrows). Thus, these results demonstrate that Ece1 is required

for vaginal immunopathogenesis *in vivo* and that hypha formation alone is insufficient to elicit hallmark immunopathology.

#### **Candidalysin-dependent innate cytokine expression is conserved between mouse**

**and human.** We also wanted to determine whether the Candidalysin-induced innate immune response observed in human vaginal epithelial cells paralleled cytokine expression in the murine vaginal mucosa *in vivo*. RNA was isolated from whole vaginas of mice challenged with WT, *ece1* $\Delta/\Delta$ , *ece1* $\Delta/\Delta$ +*ECE1*, *ece1* $\Delta/\Delta$ +*ECE1* $_{\Delta 184-279}$  and PBS sham and gene expression assessed by qPCR. Overall, cytokine gene expression patterns were similar between *in vitro* and *in vivo* samples, including Candidalysin-induced expression of the genes *Il-6*, *Cxcl2*, *Il-1a*, and *Il-1b* (Fig. 5A,C-E). There was a similar trend for expression of the genes *Cxcl1* and *Gm-csf*, although only the *ECE1* null mutant (*ece1* $\Delta/\Delta$ ) demonstrated a statistically significant reduction in cytokine gene induction (Fig. 5B,F). Unexpectedly, *G-csf* gene expression was not increased during challenge with any of the fungal strains, unlike that observed with Candidalysin treatment (Fig. 5G). In the oral cavity, *C. albicans* induces expression of the antimicrobial peptide (AMP) cathelicidin, of which the murine equivalent is the cathelicidin related AMP (CAMP) (25). Interestingly, the gene encoding for CAMP was not induced in the vagina by Candidalysin, and in fact was down-regulated similarly by all strains as compared to sham treatment (Fig. 5H). However, induction of the antimicrobial peptide  $\beta$ -defensin 3 (mBD3) gene was Candidalysin-dependent (Fig. 5I).

We also sought to determine if two inflammatory markers previously identified as associated with VVC immunopathology were regulated in a Candidalysin-dependent

manner. Expression of the gene coding for *S100a8*, a calcium-binding protein with important functions in antifungal defense and danger responses and strongly induced during *C. albicans* infection, was almost completely absent during infection with Candidalysin deletion strains (Fig. 5J) (26, 27). Similarly, the gene encoding serum amyloid A3 (*Saa3*), an inducible acute phase apolipoprotein capable of recruiting immune cells to inflammatory sites, was similarly increased in a Candidalysin-dependent fashion (Fig. 5K) (28, 29).

Lastly, we validated whether induction of several of these cytokines at the protein level by ELISA at both d 3 and d 7 p.i. was Candidalysin-dependent. Indeed, *C. albicans*-mediated secretion of IL-1 $\alpha$ , IL-1 $\beta$ , CXCL2, and S100A8 into the vaginal lavage fluid required expression of a functional Candidalysin (Fig. 6A-H). Despite increased expression of the genes encoding for IL-6, CXCL1, and GM-CSF in vaginal tissue, we were unable to demonstrably quantify these cytokines at the protein level in the lavage fluid of mice inoculated with any of the *C. albicans* strains tested (data not shown).

## DISCUSSION

In recent years, vulvovaginal candidiasis has been identified as an immunopathology, in which the host immune response, orchestrated by a series of pro-inflammatory cytokines and chemokines, actually exacerbates symptomatic disease. A landmark live-challenge study conducted by Fidel and colleagues led to this paradigm shifting hypothesis, as presence of neutrophils in the vaginal lavage fluid of women intravaginally inoculated with *C. albicans* was tightly correlated to disease

284 symptomatology (e.g. vaginal itching, burning, discomfort) (4). Ultimately, initial  
285 activation of innate immune signaling recruit neutrophils to the vaginal mucosa and  
286 experimental evidence suggests that these cells then amplify the inflammatory cascade,  
287 seemingly without reducing fungal burden (27, 30). Although several data suggest that  
288 *C. albicans* hyphal morphology is associated with immunopathology, the precise  
289 virulence attributes that enable *C. albicans* to initiate these early signaling events at the  
290 vaginal mucosa have not been well defined. The *in vitro* and *in vivo* data presented in  
291 this study identify Candidalysin as the crucial virulence factor that drives both,  
292 *C. albicans*-induced neutrophil recruitment and vaginal immunopathogenesis.

293 **The vaginal epithelial response to *C. albicans* infection provides new insight into**  
294 **immunopathological signaling.** Microarray data derived from *C. albicans*-infected  
295 human vaginal epithelial cells strongly paralleled what had been observed previously  
296 using targeted multiplex cytokine assays to determine host response to vaginal infection  
297 (6). Unsurprisingly, many of the genes uncovered by RNA-Seq as differentially  
298 regulated during murine vaginitis were not found in the human microarray datasets (11).  
299 The first explanation of this is that the human response (microarray data) is not strictly  
300 homologous with the murine response. However, given the similarity and linkage of  
301 immunopathology with neutrophil influx to the vaginal lumen in both human and murine  
302 vaginal infections, this explanation seems less likely. A more plausible explanation is  
303 that the microarray data presented here provide an epithelial-specific response that is  
304 independent of hormonal modulation or other cell-type intervention. Murine RNA-Seq  
305 data were derived from whole vaginal tissue, thus hematopoietic and stromal  
306 compartments were similarly represented (11). While each strategy offers its own

unique strengths and weaknesses, direct comparison between datasets must be done with caution. However, despite these methodological differences, there was relatively strong conservation between pro-inflammatory responses in both datasets. In both human and murine tissues, expression of phospholipase A2 (*PLA2GB4*) and prostaglandin synthase 2 (*PTGS2*) genes were both increased, suggesting activation of eicosanoid signaling at the vaginal mucosa. Indeed, this had been previously (indirectly) confirmed *in vivo* by measuring prostaglandin E2 metabolites in the vaginal lavage fluid of infected mice and human volunteers (11, 31). Increased expression of *IL-1B* and the IL-1 receptor antagonist (*IL1-RN*) genes was also identified in both datasets, suggesting that IL-1 circuit is activated in a *C. albicans*-specific manner at the vaginal epithelium. Similarly, previous work conducted by Fidel and colleagues investigating the release of S100A8/A9 alarmins in murine vaginal lavage fluid following *C. albicans* infection and immunohistological staining identified their increased expression in the vaginal epithelium (30). Expression of *S100A8/9* genes was not identified in the microarray analysis *per se*, but a gene encoding the functionally related RAGE-agonist S100P, also implicated in innate inflammation and AP1 activation, was induced following *C. albicans* challenge (32). In a similar fashion, a gene encoding the neutrophil attractant CXCL2, a downstream effector of Th17 responses, was strongly up-regulated in all datasets, suggesting that this chemokine may play a major role in attracting neutrophils to the site of infection (33).

An interesting observation from the microarray analysis was that a gene encoding the neuronal precursor of cell expressed developmentally down-regulated 9 (*NEDD9*) was increased 5.3-fold post-*C. albicans* challenge. Recently using RNA-Seq,

Liu et al. demonstrated that the *NEDD9* pathway was activated in oral keratinocytes following *C. albicans* infection (34). However, further analysis by this group, examining gene expression derived from longitudinal vaginal swabs during healthy and symptomatic vaginitis states revealed that *NEDD9* was not activated at the vaginal mucosa, but instead activated pathways were predicted to involve v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, (*ERBB2*) and platelet-derived growth factor (*PDGF-BB*) signaling in the vagina. Given these disparate results, it is unclear if *NEDD9* may play a role in activating gene expression at the vaginal mucosa in response to *C. albicans*. Interestingly, our microarray dataset (human vaginal epithelium) did not directly demonstrate increased expression of genes encoding *ERBB2* or *PDGF-BB*, but several genes coding for downstream targets of these factors were highly induced, including *JUNB*, *FOS*, *DUSP5*, *NR4A1*, *IL-8*, *IL-1B*, *TNFAIP3*, and *EGR1*, among others. Many of these same downstream targets were previously found to be induced in oral epithelial cells on the protein level (35). Therefore, *in vitro* infection of A431 cells largely mimics responses observed in both oral epithelial cells and during clinical vaginitis.

Another striking observation was the strong transcriptional up-regulation of the gene encoding EGF ligand heparin-binding EGF-like growth factor (*HB-EGF*, ~40-fold) and the gene coding for the enzyme heparin sulfate 3-O-sulfotransferase 1 (*HS3ST1*), suggesting the potential presence of heparin sulfate at the vaginal mucosa. Indeed, recent work by Yano et al. demonstrated that heparin sulfate can be recovered from the vaginal lavage fluid of mice and that its presence is enhanced by exogenous estrogen administration (36). Interestingly, treatment of recovered vaginal fluid with heparinase



restored the capacity of PMNs to kill *C. albicans in vitro*, suggesting that heparin sulfate may phenotypically alter or physically inhibit neutrophil-fungus interaction. One potential mechanism was presented whereby heparin sulfate outcompetes the fungal surface antigen Pra1 for its natural ligand Mac1 present on the surface of neutrophils to prevent killing of the fungus (36, 37). Although the precise role of heparin sulfate at the vaginal mucosa remains unclear, it is often up-regulated in other epithelial or epidermal tissues in response to damage, functioning in the tissue repair process (38). Therefore, the capacity of *C. albicans* hyphae and Candidalysin to damage epithelia and potentially elevate free vaginal heparin sulfate may indirectly contribute to a fungal fitness strategy to defend against PMN-mediated clearance at the mucosal surface. Collectively, these results may help explain why neutrophils are ineffective at reducing fungal burden during vaginitis, despite being robustly recruited to the vaginal lumen.

**Candidalysin: the key fungal factor driving damage and vulvovaginal immunopathogenesis.** Previous data generated from our laboratories have demonstrated that both oral and vaginal epithelial cells can differentially sense and respond to yeast and hyphal forms of *C. albicans* (6, 8, 9). However, modestly different signaling mechanisms, cytokine secretion, and hyphal burden thresholds differentiate responses in oral and vaginal epithelial cells. For example, previous studies by Moyes et al. determined that the A431 vaginal cell line responds to *C. albicans* challenge in a similar fashion to TR146 oral cells, albeit at fungal burdens roughly one log greater (6). Moreover, the cytokines CCL20, G-CSF, and IL-6 were robustly secreted by oral epithelia in response to *C. albicans* infection. However, these responses were comparatively reduced or not observed during *in vitro* infection of vaginal epithelium or

at the murine vaginal mucosa. These differences in responses could be due to the abovementioned apparent elevated signaling threshold in vaginal cells or may represent site specific fine-tuning of mucosal immunity—a hypothesis supported by the observed differences in transcription factors activated in the two cell types (6, 8). Furthermore, some cytokines (e.g. IL-6) were induced by treatment with Candidalysin alone but not during infection with *C. albicans*. It is likely that Candidalysin concentration plays a major factor in its lytic and immunostimulatory function and currently it is unclear what general or microniche concentrations of Candidalysin are present in an *ex vivo* or *in vivo* setting or what other host or fungal factors it interacts with during infection.

The hypothesis that epithelial surfaces can discriminate between yeast and hyphal forms of *C. albicans* has been established by linking hypha formation with the capacity to damage epithelial surfaces (6, 8). These observations have recently been extended into *in vivo* findings, as a similar phenomenon also appears to be true at the murine vaginal mucosa (9, 39). Indeed, hypha-deficient mutants of *C. albicans* fail to induce pro-inflammatory signaling, despite very high levels of colonization. One key feature explaining these dichotomous responses is lack of elicited epithelial damage and subsequent danger-associated molecular pattern (DAMP) release by hypha-deficient strains. This hypothesis is further supported by studies examining the role of the NLRP3 inflammasome, a danger-sensing molecular complex, in mediating inflammation at both oral and vaginal mucosal surfaces (40, 41). The use of NLRP3-/- mice during oral and vaginal candidiasis has demonstrated that neutrophil migration and pro-inflammatory signaling is reduced in these animals, presumably due to a defect in the ability to recognize and respond to DAMP signals (11, 25, 42, 43). As for vaginal

candidiasis, a population-level genetic study revealed that the 12/9 genotype was significantly associated with high levels of NLRP3 effector cytokines found in the vaginal lavage fluid of women with recurrent VVC (RVVC), suggesting the recognition of DAMP signals likely prime the epithelial pro-inflammatory response (44). This concept of linking fungal pathogenicity to damage was further supported by findings from Schönherr et al. in which the virulence of clinical isolates of *C. albicans* was directly correlated with their capacity to induce oral mucosal insult (43). Notably, only the expression of *ECE1/Candidalysin* was strongly correlated with damage and pathogenesis in several (but not all) *C. albicans* isolates. However, it is likely that simultaneous and combined expression of several attributes (e.g. hyphae and Candidalysin) is required for full virulence.

Given that the presence of Candidalysin is sufficient to induce damage to the vaginal mucosa and elicits inflammasome effector responses (i.e. IL-1 $\beta$ ), it is possible that Candidalysin serves as a fungal DAMP capable of inflammasome activation. Typically, inflammasome activation requires two independent steps, the first of which is ligation of pattern recognition receptors that prime the response and a second damage step that activates the response (40). Obviously the lytic property of Candidalysin satisfies the second step but activation of the first step would likely depend on other cellular factors of *C. albicans* (e.g. cell surface components). However, results from Moyes et al, in which Candidalysin was mutated to contain a C-terminal alanine–alanine motif (*Ece1-III*<sub>62–93AA</sub>) retained immunostimulatory function in the absence of lytic activity, suggesting that structural recognition of this peptide may be sufficient to also

prime inflammasome activity or other inflammasome-independent inflammatory responses (16). Investigations are currently underway to address these possibilities.

In summary, this study demonstrates that Candidalysin secretion is critical for the induction of immunopathological signaling at the vaginal mucosa, and that these responses are largely conserved at both human and murine epithelial surfaces. Furthermore, our findings decouple hypha formation from disease symptomatology and clearly link vaginitis immunopathogenesis with Candidalysin production and its capacity to directly damage the vaginal mucosa. In light of these findings, studies designed to determine the mechanistic interaction of Candidalysin with the vaginal epithelium are warranted. Therapeutic strategies to either neutralize Candidalysin itself, inhibit its expression, or block downstream host signaling pathways may offer a unique opportunity to more quickly arrest symptomatology of this most prevalent human fungal infection.

## **MATERIALS AND METHODS**

**Ethics statement.** The animals used in this study were housed in AAALAC-approved facilities located at the University of Tennessee Health Sciences Center (UTHSC) in the Regional Biocontainment Laboratory (RBL). The UTHSC Animal Care and Use Committee approved all animals and protocols. Mice were given standard rodent chow and water *ad libitum*. Mice were routinely monitored for signs of distress, including noticeable weight loss and lethargy.

**Cell lines, strains and primers.** The A431 human vulvar epidermoid carcinoma cell line was used in this study. All *C. albicans* strains used, including Candidalysin deletion mutants, are those described by Moyes, et al (16, 45). All primers used for quantitative PCR (qPCR) are listed in Table S2.

**Microorganism growth.** *C. albicans* strains were maintained as glycerol stocks stored at -80°C. A small amount of stock was spread onto yeast peptone dextrose (YPD) agar and incubated at 30°C for 48 h to obtain isolated colonies. A single colony was transferred to 10 mL of YPD liquid medium and incubated at 30°C with shaking at 200 rpm for 16 h prior to vaginal infection.

**Microarray analysis.** Reconstituted human vaginal epithelia (RVE: 5-day) created using the A431 cell line were purchased from SkinEthic Laboratories (France) and used as previously described (6). RNA was isolated from RVE infected with *C. albicans* SC5314 for 6 and 24 h or an equal volume of PBS using the GenElute total mammalian RNA miniprep kit (Sigma, UK) and trace genomic DNA removed using the Turbo DNase-free kit (Ambion, UK). For microarray analysis, RNA was amplified using the MessageAmp Premier RNA Amplification Kit (Ambion, UK) and hybridized onto U133a 2.0 gene chips (Affymetrix, UK) after fragmentation by metal-induced hydrolysis into 35-200 nucleotide fragments according to standard protocols. Chips were scanned (Affymetrix GeneChip Scanner 3000) and assessed using Affymetrix Command Console (AGCC) software suite. This data was statistically analyzed using the

465 Bioconductor R package, PIANO. Gene Ontology and pathway analysis was performed  
466 using both PIANO and DAVID (46, 47).

467

468 **Cytokine release.** A431 vaginal epithelial cells were cultured in Dulbecco's Modified  
469 Eagle Medium Nutrient Mixture + L-glutamine (Life technologies) supplemented with  
470 10% (v/v) heat-inactivated fetal bovine serum (Life technologies) and 1% (v/v) penicillin-  
471 streptomycin (Sigma) at 37°C, 5% CO<sub>2</sub>. Candidalysin peptide  
472 (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK) was purchased from Peptide Protein  
473 Research Ltd (UK). Prior to Candidalysin challenge, confluent A431 epithelial cells were  
474 serum-starved overnight and all experiments were carried out in serum-free DMEM  
475 medium. Cells were incubated with Candidalysin (prepared as a 10 mg/mL stock in  
476 sterile water)) at doses of 1.5, 3, 15 and 70 µM for 2 h at 37°C in 5% CO<sub>2</sub>. Sterile water  
477 (vehicle only) controls were also included. Culture supernatants were then isolated and  
478 human IL-1α, IL-1β, IL-6, IL-8, GM-CSF, and G-CSF quantified by Magnetic Luminex  
479 Performance Assay (Biotecne) and Bio-Plex 200 System (BioRad) according to the  
480 manufacturer's instructions.

481

482 **Epithelial cell damage assay.** Damage to epithelial monolayers following a 24 h  
483 challenge with Candidalysin was determined by quantification of lactate  
484 dehydrogenase activity in cell culture supernatants using a CytoTox 96 non-radioactive  
485 cytotoxicity assay (Promega) according to the manufacturer's instructions as previously  
486 described (16). Porcine lactate dehydrogenase (Sigma) was used to create the  
487 standard curve.

488

489 **Preparation of protein extracts.** Epithelial cells were lysed using a modified RIPA  
490 buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1%  
491 sodium deoxycholate, 0.1% SDS) containing protease (Sigma-Aldrich) and  
492 phosphatase (Perbio Science) inhibitors. Crude lysates were cleared by centrifugation  
493 at 4°C and protein concentration estimated by BCA assay (Thermo Scientific) according  
494 to the manufacturer's instructions.

495

496 **SDS PAGE and Western blotting.** Proteins were resolved by electrophoresis on 12%  
497 SDS PAGE gels using a mini-protean tetra cell system (BioRad). Electrophoresed  
498 proteins were transferred to nitrocellulose membrane (BioRad) using a mini-transblot  
499 electrophoretic transfer cell (BioRad). p-DUSP1/MKP1 (S359) and c-Fos rabbit  
500 monoclonal antibodies were purchased from Cell Signaling Technologies. Actin (clone  
501 C4) mouse monoclonal antibody was purchased from Millipore. Peroxidase-conjugated  
502 Affinipure Goat anti-mouse and anti-rabbit IgG secondary antibodies were purchased  
503 from Jackson Immune Research. Membranes were blocked in 1 × TBS (Severn  
504 Biotech) containing 0.001% (v/v) tween-20 (Acros Organics) and 5% (w/v) fat free milk  
505 powder (Sainsbury's). Primary antibodies diluted (1:1000 or 1:10,000 as suggested by  
506 manufacturer) in TBS-tween and 5% milk (c-Fos), or TBS-tween and 5% bovine serum  
507 albumin (p-DUSP1/MKP1) were added and membranes incubated overnight at 4°C with  
508 gentle shaking. Following incubation, membranes were washed with 1 × TBS containing  
509 0.01% (v/v) tween-20, diluted (1:10,000) HRP-conjugated secondary antibody added  
510 and membranes incubated for 1 h at room temperature. Membranes were washed as

described and exposed to Immobilon Western Chemiluminescent HRP substrate (Millipore) prior to visualisation by exposure to film (GE Healthcare). Alpha-actin was used as a loading control.

**Murine model of vaginal candidiasis.** A murine model of *Candida* vaginitis was utilized as described previously (9, 11, 48). Female 6-8 week old C57BL/6 mice were purchased from Charles River laboratories and housed in isolator cages mounted onto ventilated racks. Mice were administered 0.1 mg of estrogen ( $\beta$ -estradiol 17-valerate; Sigma) dissolved in 0.1 mL sesame oil subcutaneously 72 h prior to inoculation with *C. albicans*. Stationary-phase cultures of *C. albicans* strains were washed three times in sterile, endotoxin-free phosphate-buffered saline (PBS) and resuspended in a 0.2  $\times$  volume of PBS. Cell suspensions were diluted, counted on a Neubauer hemocytometer, and adjusted to  $5 \times 10^8$  CFU/mL in sterile PBS. Estrogen-treated mice were intravaginally inoculated with 10  $\mu$ L of the standardized blastoconidial cell suspension, generating an inoculum size of  $5 \times 10^6$  blastoconidia. At d 3 and/or d 7 p.i. mice underwent vaginal lavage with 100  $\mu$ L of PBS. Resultant lavage fluids were spiked with 1  $\mu$ L of 100  $\times$  EDTA-free protease inhibitors (Roche) and kept on ice until processing for immunopathological markers. After sacrifice, vaginal tissue was surgically excised and stored for downstream analyses. All animal experiments were conducted with n=4 mice per group, repeated, and data combined unless noted otherwise.

**Assessment of fungal burden and vaginitis immunopathology.** All immunopathological markers were assessed as described previously (9). (i) Lavage



fluid was serially diluted 10-fold using the drop-plate method, plated onto YPD agar containing 50 µg/mL chloramphenicol, plates incubated for 24 h at 37°C, and the resulting colonies enumerated. CFU/mL values per group are reported as medians. (ii) Lavage fluid (10 µL) was smeared onto glass slides and stained by the Papanicolaou technique to assess polymorphonuclear leukocyte (PMNs) recruitment (small, blue, cells with multi-lobed nuclei). PMNs were counted in 5 non-adjacent fields by standard light microscopy using a 40X objective and values reported as mean + standard error of the mean (SEM). (iii) Murine IL-1 $\alpha$ , IL-1 $\beta$ , CXCL2, and S100A8 was assessed in clarified, diluted (1:20-1:100) vaginal lavage fluid using commercial enzyme-linked immunosorbent assays (eBioscience, R&D Systems) according to manufacturer's protocol. Results are reported as the mean + SEM. (iv) Lactate dehydrogenase (LDH) activity was measured in clarified, diluted (1:100) lavage fluid using the commercial available CytoTox 96 nonradioactive cytotoxicity assay (Promega). Results are reported as the mean + SEM.

**Isolation of RNA from vaginal tissue.** RNA was extracted from whole vaginas as described previously (11). At d 3 p.i., vaginal tissue was surgically excised, immediately placed into RNALater (Thermo Fisher), and incubated at 4°C overnight. The following day, tissues were transferred to TRI Reagent (Sigma), finely minced with scissors, mechanically homogenized (Pro Scientific), and centrifuged at 12,000  $\times$  g for 10 min at 4°C. RNA was isolated by chloroform-ethanol precipitation and the pellet resuspended in nuclease-free water according to TRI Reagent instructions. RNA concentration was measured by spectroscopy at A260/280 and integrity verified by 3-(N-

morpholino)propanesulfonic acid (MOPS) gel electrophoresis to visualize intact 18s and 28s rRNA bands.

**qRT-PCR analysis.** RNA from vaginal tissue was isolated as described above. RNA concentrations were equalized amongst samples and 200 ng aliquots were treated with RNase-free DNase according to the manufacturer's instructions (Thermo Scientific). RNA was reverse transcribed using random hexamers and the RevertAid kit according to the manufacturer's protocol (Thermo Scientific). Proprietary primer sets spanning exon-exon junctions were ordered from IDT for murine *Il-6*, *Cxcl1*, *Cxcl2*, *Il-1a*, *Il-1b*, *Gm-csf*, *G-csf*, *Camp*, *S100a8*, *Saa3*, *Defb3*, and *Act1b* (**Table S2**). All primers were used at the manufacturer's recommended concentrations along with 2 × Maxima Sybr Green mix (Bio-rad) to amplify 20 ng of cDNA. qPCR reactions were monitored and analyzed with the Applied Biosystems 7500 platform and associated software. Expression levels of target genes in infected mice were compared to a reference gene (*ACT1B*) and naïve controls using the  $\Delta\Delta C_t$  method as described previously (49).

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580

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584 strains. Experimental design was conducted by DLM, JRN, BH and BMP. JPR, HMEW,  
585 DLM, SS, KSB, SLT, and GEP performed all experimental techniques and data  
586 analysis. All authors aided in experimental critique and manuscript preparation.

587

588

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## FIGURE LEGENDS

**Figure 1. Global differential gene expression induced during *C. albicans* infection of Reconstituted Vaginal Epithelium (RVE).** RVE were challenged with *C. albicans* or mock infected with PBS for 6 or 24 h and differential gene expression was assessed by microarray analysis. Volcano plots depicting log2 fold expression changes of genes between **(A)** 6 h and **(B)** 24 h time points following challenge with *C. albicans* or PBS sham. **(C)** After adjusting for p-value and false discovery rate (fdr,  $p < 0.01$ ) gene, Venn-diagram plot depicts absolute number of genes expressed between *C. albicans* at 6 h (red) and 24 h (green).

**Figure 2. Host signaling pathways predicted to be activated during *C. albicans* infection of RVE.** Based on differential gene expression, KEGG pathway analysis using the DAVID web-based package revealed several host pathways predicted to be significantly ( $p < 0.0001$ ) activated at **(A)** 6 h or **(B)** 24 h. Pathways are listed in order of highest probability of activation. Significance was assessed using DAVID statistical package via ANOVA analysis.

**Figure 3. Candidalysin is sufficient to induce cellular damage and proinflammatory responses in vaginal epithelial cells. (A)** A431 vaginal epithelial

cells were exposed to Candidalysin (70, 15, 3 and 1.5  $\mu$ M) for 24 h and cellular damage quantified by LDH assay. Data are presented as fold change relative to vehicle control. Statistics are applied relative to the vehicle control (n=3 biological repeats). **(B)** Western blot analysis of the vaginal epithelial response to different concentrations of Candidalysin. Epithelial cell lysates (20  $\mu$ g total protein) were probed with anti c-Fos and anti p-MKP1 antibodies. One representative blot presented (from n=3 biological repeats). **(C-H)** Quantification of cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , G-CSF, GM-CSF, IL-6, and IL-8) secreted from vaginal epithelial cells in response to different concentrations of Candidalysin. Statistics are applied relative to the vehicle control (n=3 biological repeats). Graphs are plotted as the mean + SEM. A and C-H: Statistical significance was calculated using one-way ANOVA and Dunnet's post-test. \*\*\* p < 0.001, \*\* p < 0.01, \* p < 0.05.

**Figure 4. Candidalysin is required for neutrophil recruitment and mucosal damage in a murine model of vulvovaginal candidiasis.** Groups of estrogen-treated C57BL/6 mice (n=4) were intravaginally challenged with WT (black bars), *ece1* $\Delta/\Delta$  (dark gray bars), *ece1* $\Delta/\Delta$ +*ECE1* $\Delta_{184-279}$  (white bars), and *ece1* $\Delta/\Delta$ +*ECE1* (light gray bars) strains of *C. albicans* and vaginal lavage fluid assessed longitudinally at d 3 and d 7 for **(A, B)** fungal burden, median; **(C, D)** PMNs, mean + SEM; **(E, F)** the damage biomarker LDH, mean + SEM. **(G)** Papanicolaou staining was performed on smears made from vaginal lavage fluid to assess PMN influx (yellow arrows) and hypha formation (green arrows) at d 3 and d 7 p.i. and are representative images. All inoculation groups were

performed in duplicate and data combined. A-F: Statistical significance was calculated using a one-way ANOVA and Tukey's post-test. \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$ .

**Figure 5. Candidalysin is required for pro-inflammatory cytokine expression in the murine vagina.** Groups of estrogen-treated C57BL/6 mice (n=4) were intravaginally challenged with WT (black bars), *ece1* $\Delta/\Delta$  (dark gray bars), *ece1* $\Delta/\Delta$ +*ECE1* $\Delta_{184-279}$  (white bars), and *ece1* $\Delta/\Delta$ +*ECE1* (light gray bars) strains of *C. albicans*, whole vaginal tissue excised at d 3 p.i., and extracted RNA processed for qPCR analysis. Genes chosen for qPCR included those previously identified as being induced by candidalysin or *C. albicans* during in vitro or in vivo challenge, including: **(A)** *Il-6*, **(B)** *Cxcl1*, **(C)** *Cxcl2*, **(D)** *Il-1a*, **(E)** *Il-1b*, **(F)** *Gm-csf*, **(G)** *G-csf*, **(H)** *Camp*, **(I)** *Defb3*, **(J)** *S100A8*, and **(K)** *Saa3*. All genes were internally compared to the *Actb* housekeeping gene and to mock-infected controls using the  $\Delta\Delta C_t$  method. Graphs are plotted as the mean normalized fold expression + SEM. Statistical significance was calculated using a one-way ANOVA and Tukey's post-test. \*\*  $p < 0.01$ , \*  $p < 0.05$ .

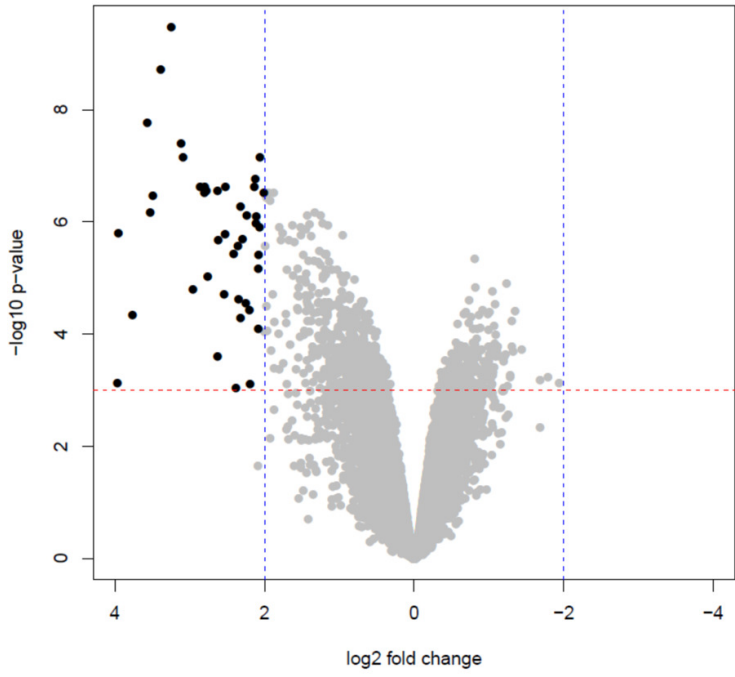
**Figure 6. Candidalysin is required for release of hallmark proinflammatory cytokines and chemokines into the vaginal lavage fluid during murine vaginitis.** Groups of estrogen-treated C57BL/6 mice (n=4) were intravaginally challenged with WT (black bars), *ece1* $\Delta/\Delta$  (dark gray bars), *ece1* $\Delta/\Delta$ +*ECE1* $\Delta_{184-279}$  (white bars), and *ece1* $\Delta/\Delta$ +*ECE1* (light gray bars) strains of *C. albicans* and vaginal lavage fluid assessed longitudinally by ELISA at d 3 and d 7 p.i. for inflammatory markers, including **(A, B)** IL-1, **(C, D)** IL-1, **(E, F)** *Cxcl2*, **(G, H)** *S100a8*. All inoculation groups were performed in

811 duplicate and data combined. Graphs are plotted as the mean + SEM. Statistical  
812 significance was calculated using a one-way ANOVA and Tukey's post-test. \*\*\*  $p <$   
813 0.001, \*\*  $p < 0.01$ , \*  $p < 0.05$ .

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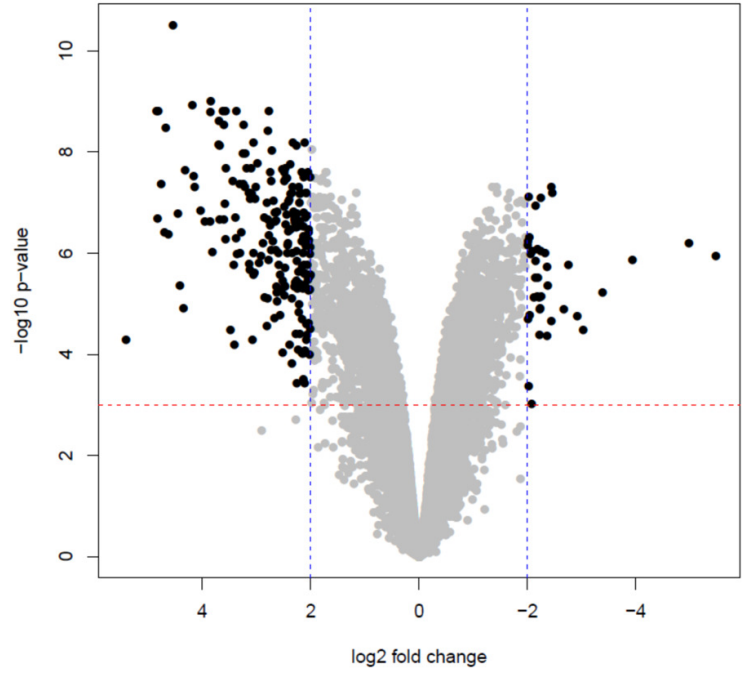
**A**

t6\_candida - t6\_pbs



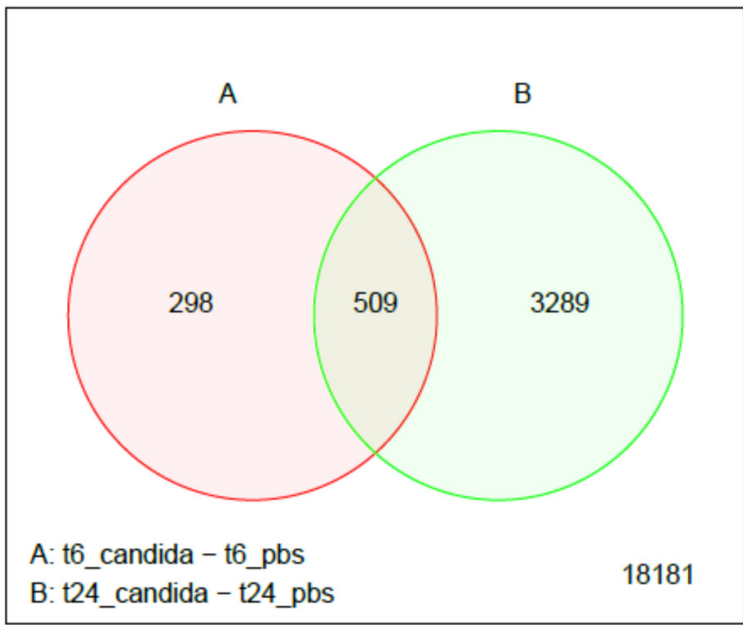
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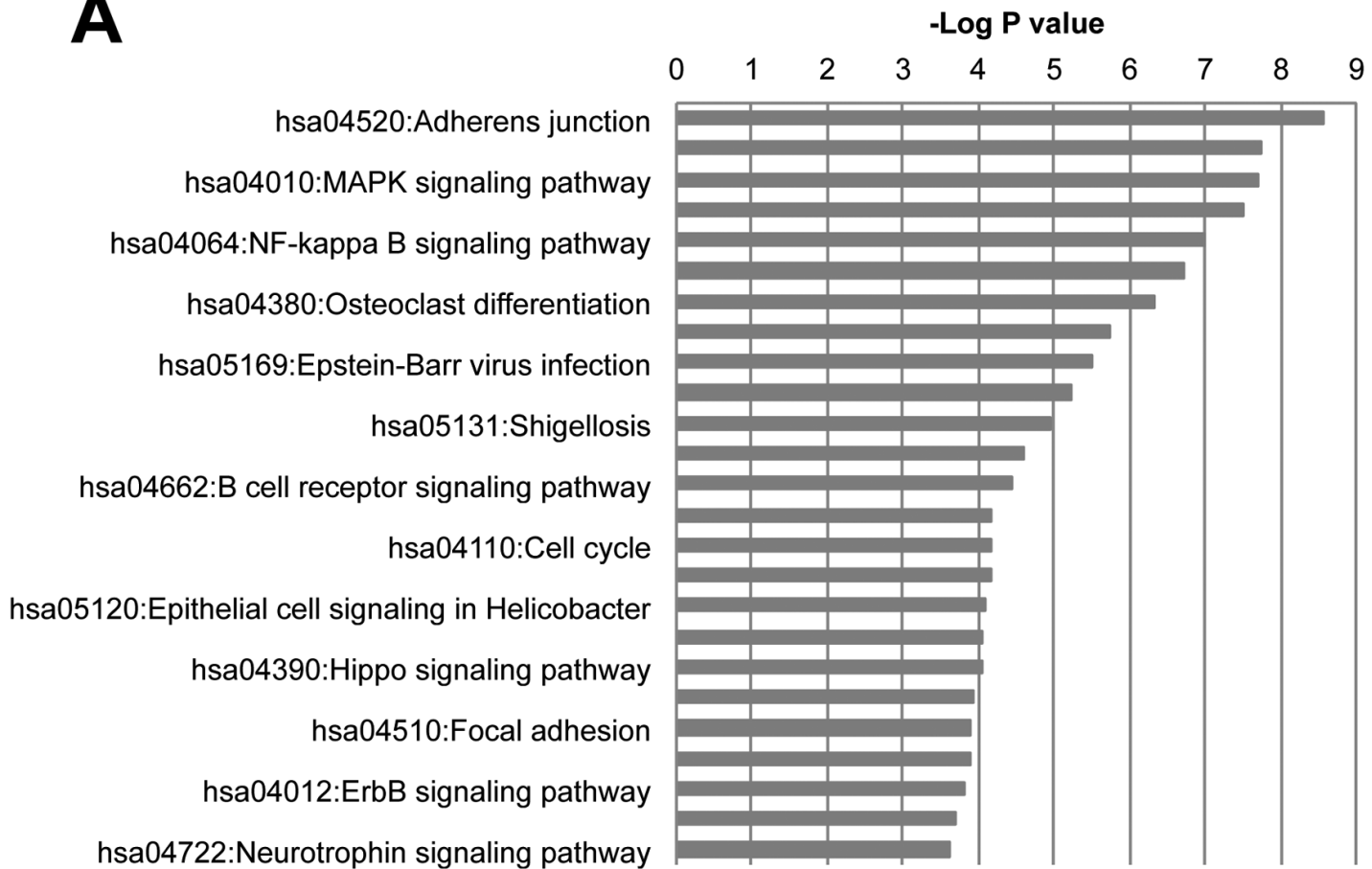
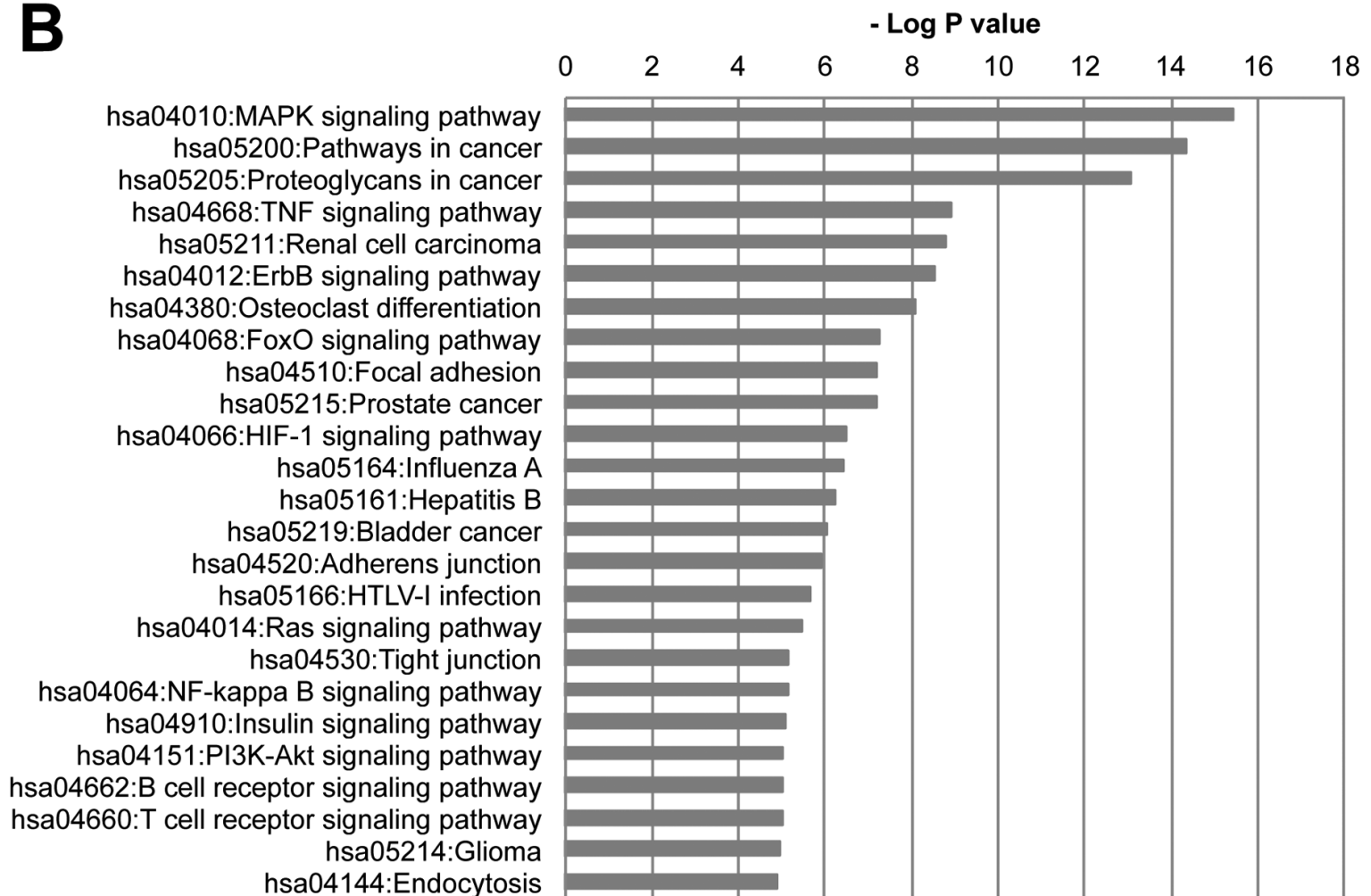
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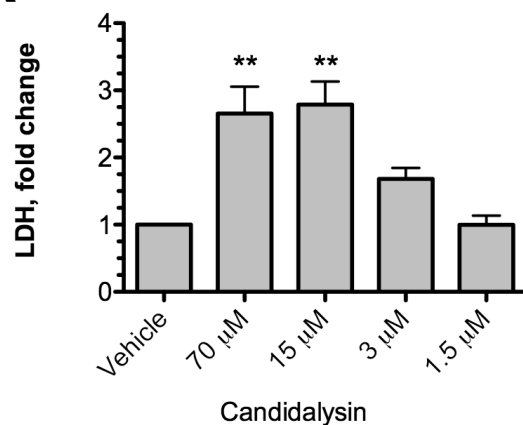
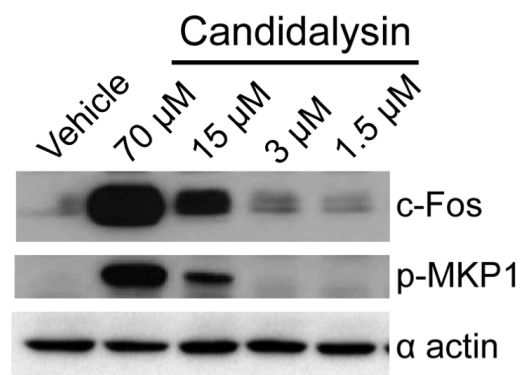
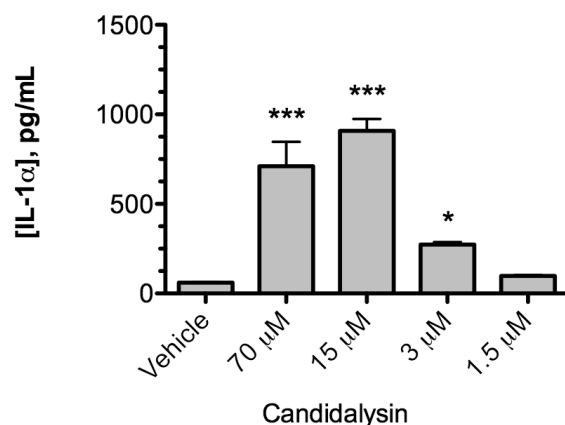
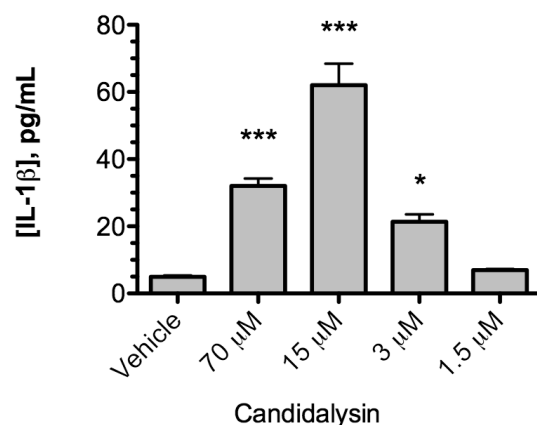
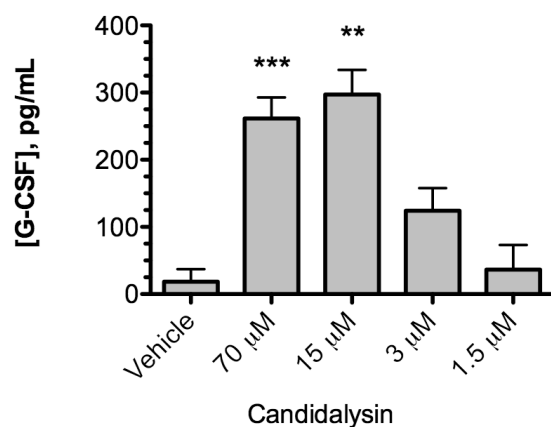
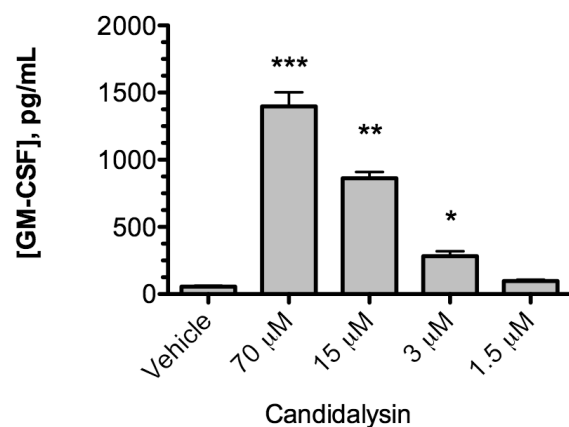
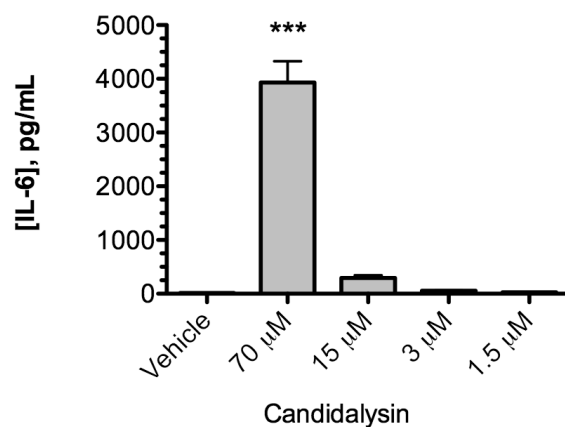
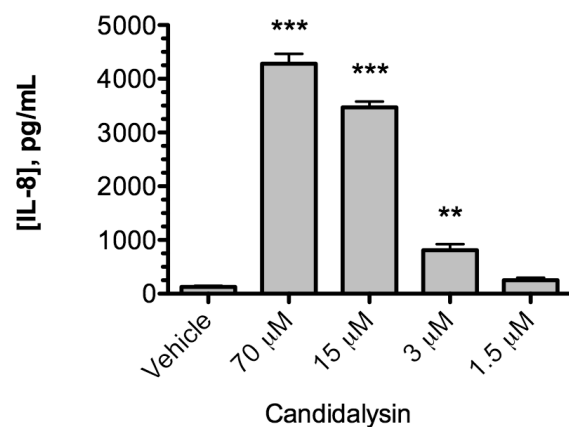
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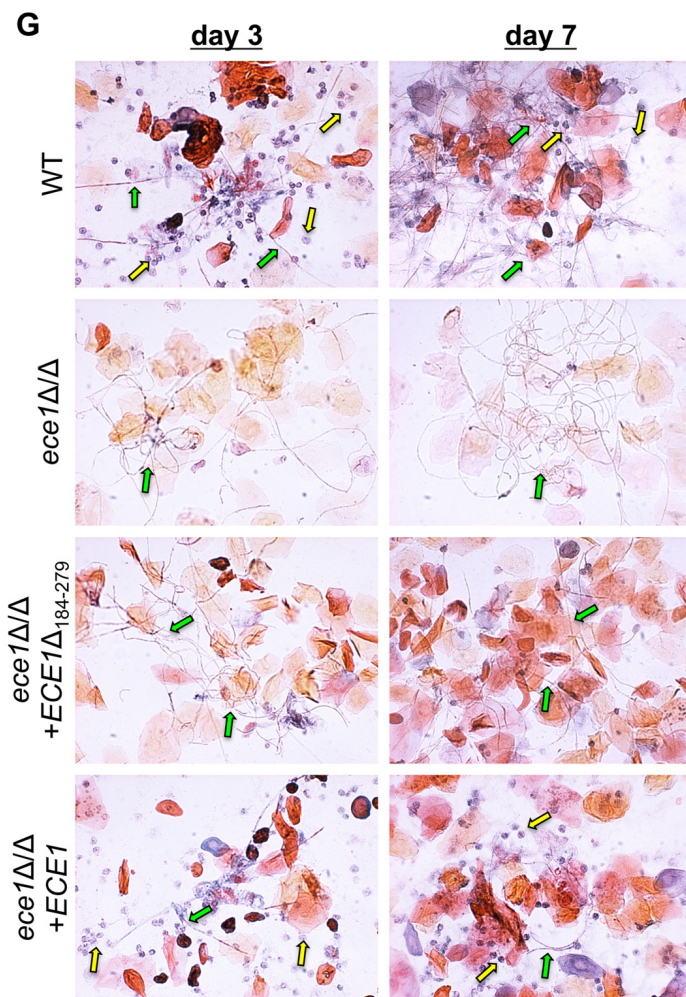
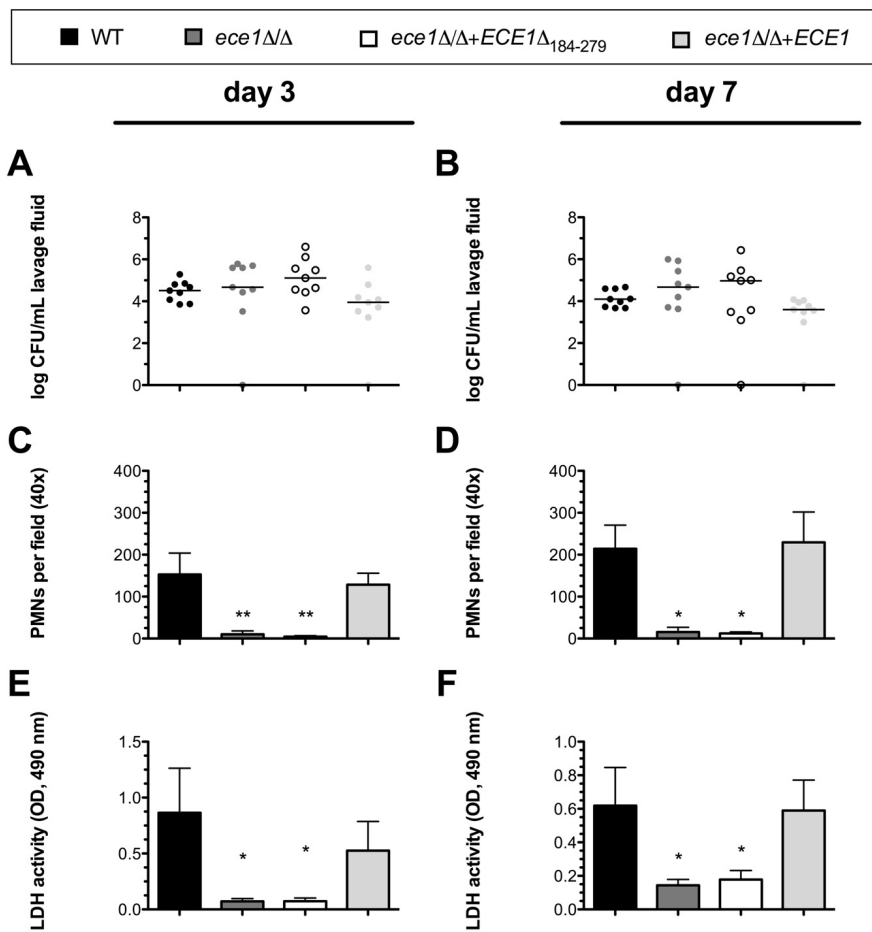
Venn diagram (p-value adjustment: fdr, p<0.001)

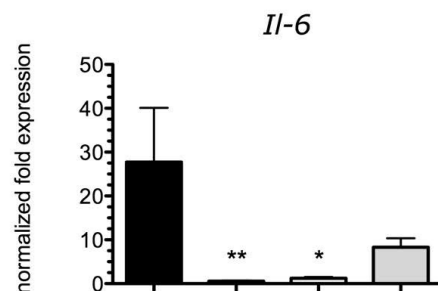
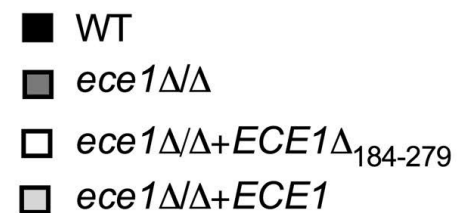
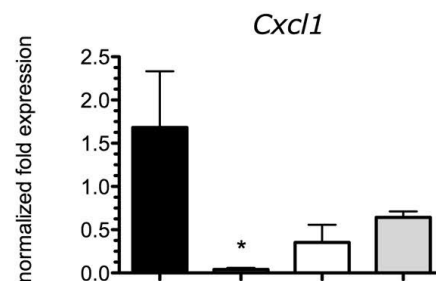
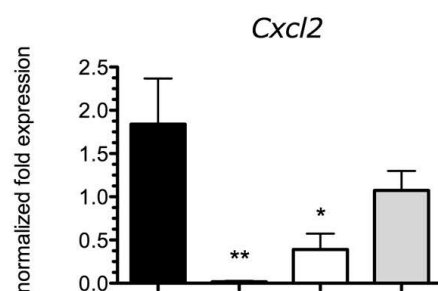
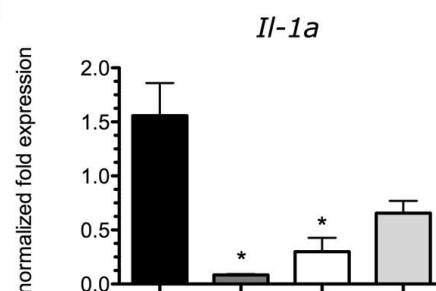
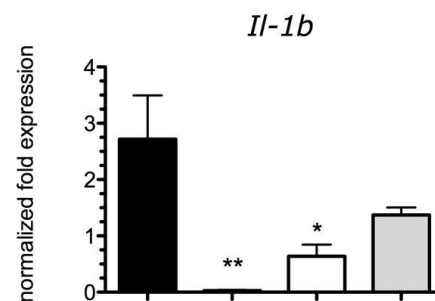
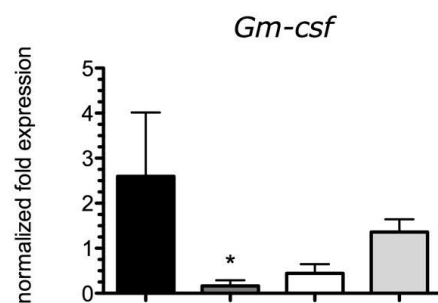
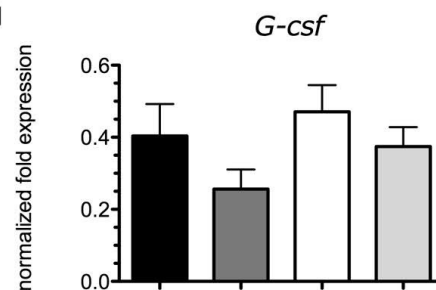
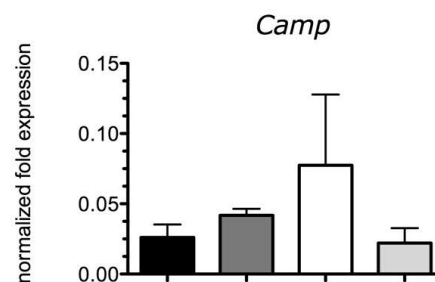
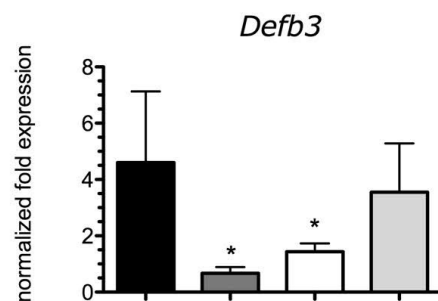
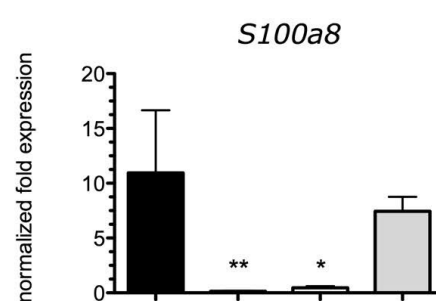
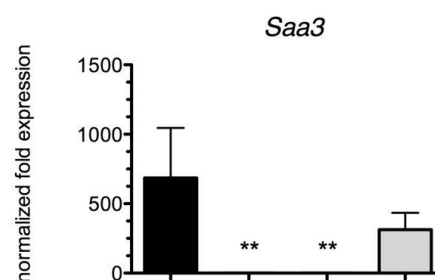


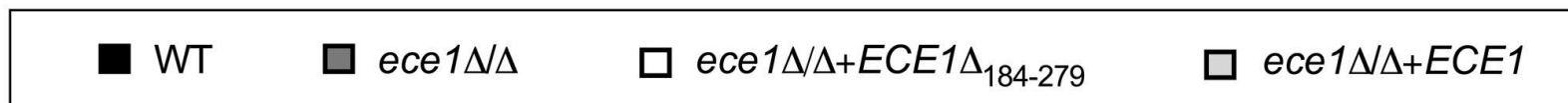
**A****B**



**A****B****C****D****E****F****G****H**

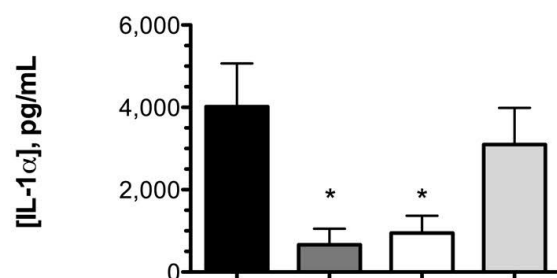


**A****B****C****D****E****F****G****H****I****J****K**



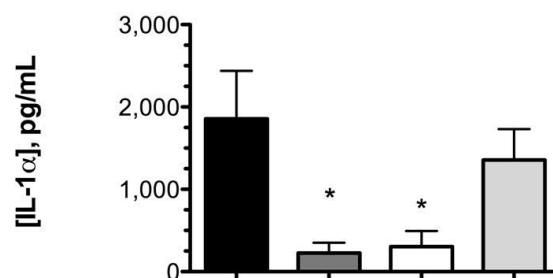
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A

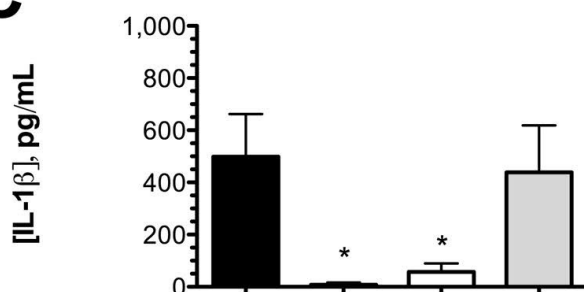


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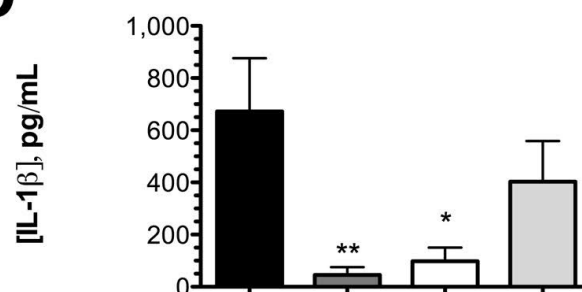
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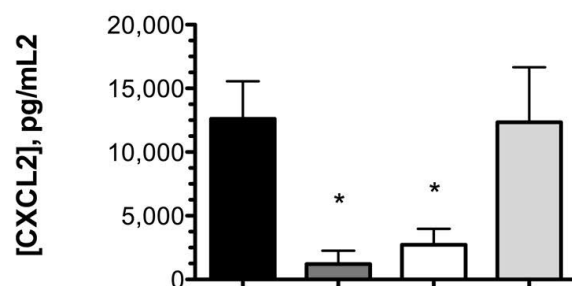
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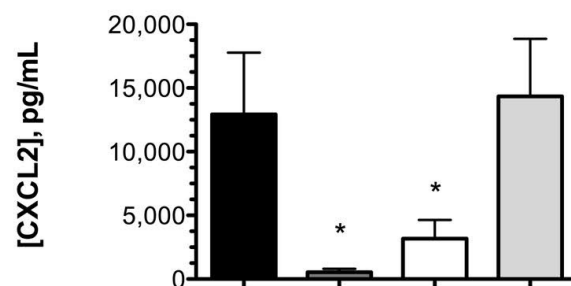
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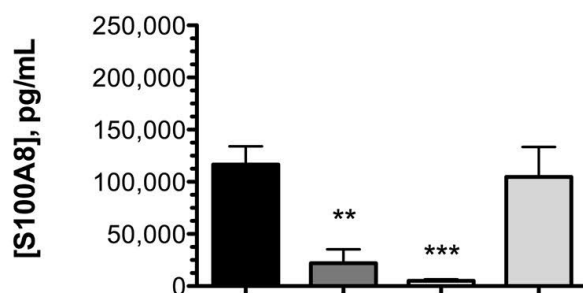
E



F



G



H

